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THE STUDY OF THE PREVALENCE OF ATYPICAL MYCOBACTERIA IN EGYPT

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THE STUDY OF THE PREVALENCE OF ATYPICAL MYCOBACTERIA IN EGYPT

The atypical mycobacteria involved in pulmonary and other human diseases, produce a disease which simulates tuberculosis and is very much like it clinically, However by culture, they show several differentiating characteristics.

These mycobacteria are acid and alcohol fast bacilli, but differ from virulent tubercle bacilli in their morphology, pigmentation, and, notably, in lacking the characteristic pathogenicity for certain animal species. Many of these mycobacteria produce self healing lesions in various organs. Few strains may be slightly pathogenic to guinea pigs.

In our study of the prevalence of atypical mycobacteria in Egypt, we are aiming at examining the properties and significance of a collection of strains of these organisms met with in the central T.B. laboratory from all over the country.

Most strains were isolated from patients with symptoms and findings simulating tuberculosis as well as from patients suffering from actual tuberculosis.

They grow in pure culture or together with virulent tubercle bacilli. Both groups resemble each other morphologically

The atypical mycobacteria are usually very short or very long and are well dispersed in the stained preparations.

These mycobacteria are also distinguished by their pigmentation which ranges from cream to lemon yellow or orange, White, pink or red colours may be exhibited.

By guinea pig inoculation they produce limited or progressive fatal disease. However they may produce granulomatous lesions, self healing, in various organs and have been detected in tissues after infection.

In guinea pig inoculation, young male guinea pigs weighing about 250-350 gm. are used. Primary cultures of the atypical mycobacteria grown on egg medium are emulsified in saline for inoculation into the thigh of the guinea pig. The inoculum is standardised to 0.1 mg. moist weight. The animals are killed six weeks after inoculation.

Methods:

Collection of strains of atypical mycobacteria: During the period from September 1975 to August 1976 we received a big number of samples from Cairo. Twelve centres distributed in different districts of the town served in providing our laboratory with the pathological material.

The Guisa centres continued in supplying our laboratory with the samples.

In addition to the seven centres situated in Kafr el Sheikh, Dosouk, Shebein, Biala, Ismailia, Port Said and Zagazig, seven other centres supplied our laboratory with the pathological material. These centres are situated at Shobrahour, Menouf, Ashmoun, Fakous, Minia el Kamh, Manzala and a second centre in Kafr el Sheikh.

Proper collection and preparation of the specimens is very important in this work. Clean sterile containers were used and saliva samples were avoided.

Although the smear is the least sensitive method for the bacteriologic study of mycobacteria and cannot differentiate the type of acid fast bacilli still we used it as a rapid means for diagnosis as it takes weeks to get the result by culture and animal inoculation.

During this period about 9500 samples of sputum were received from Cairo and Guiza centres and were cultured for the tubercle bacillus. 15.62 per cent were positive. 22 strains of atypical mycobacteria were isolated giving an incidence of 0.19 per cent.

About 72.3 per cent of patients who submit samples from Cairo and Guiza centres are males and 27.7 per cent were females.

In Cairo centres about 76.77 per cent of the positives are males and about 22.33 per cent are females. Meanwhile about 69.9 per cent of the positive cultures from Guiza centres are derived from male patients and 30.1 per cent from female patients.

The sex distribution of patients yielding atypical mycobacteria in Cairo and Guiza centres is shown as follows:

CENTRE	TOTAL	MALE	FEMALE
Cairo	15	12	3
Guiza	7	5	2
Total	22	17	5

The positive cultures of sputum samples received from Cairo centres and their sex distribution are presented in the following table:

CENTRE	TOTAL	MALE +ve	PERCENT	FEMALE +ve	PERCENT
B. Sharia	583	101	17.32	42	7.2
A. Maher	1442	150	10.40	35	2.24
Khalifa	1426	126	8.83	53	3.71
Abassia	342	28	8.18	9	2.63
Boulak	432	87	20.41	17	3.93
El Marg	663	147	22.17	41	6.18
S. Zeinab	1139	128	10.79	44	3.86
Toura	88	23	26.19	—	—
Materia	226	36	15.92	11	4.86
Nafesia	132	6	4.54	—	—
Helwan	64	13	20.31	7	10.93

The sex distribution of the isolated atypical mycobacteria in Cairo centres is shown as follows:

CENTRE	CULTURE +ve	ATYPICAL STRAINS	PERCENT	MALE	FEMALE
A. Maher	185	2	1.08	2	—
Boulak	104	1	0.95	1	—
Khalifa	179	2	1.11	1	1
B. Sharia	143	3	2.9	3	—
El Marg	188	2	1.06	1	1
Materia	47	1	2.13	1	—
S. Zeinab	167	1	0.66	-	1

No atypical mycobacteria were isolated from Abassia, Helwan, or Toura centres.

In Guiza centres the sex distribution among the positive cultures is presented as follows:

CENTRE	TOTAL	MALE +ve	PERCENT	FEMALE +ve	PERCENT
Guiza D.	1477	89	6.026	40	2.707
Guiza S.	398	69	17.23	27	6.78
Imbaba	1109	113	10.01	50	4.60

The isolated strains of atypical mycobacteria and their sex distribution in Guiza centres are as follows:

CENTRE	+ve	ATYPICAL	%	MALE	FEMALE
Guiza D.	129	2	1.55	2	—
Imbaba	163	5	3.06	3	2

No atypical mycobacteria have been isolated from Guiza S. centre.

About 2700 sputum samples were received from Kafr el Sheikh, Dosouk, Shebein, Biala, Ismailia, Port Said, Zagazig and the new centres which added to our supply from Shobrahoor, Menouf, Ashmoun, Fakous, Minia el Kamh and Manzala. 30.2 per cent of these samples were positive by culture. Six strains of atypical mycobacteria were isolated giving an incidence of 0.8 per cent.

The sputum samples received during this period from these centres and positive cultures from both males and females are as follows:

CENTRE	TOTAL	MALE +ve	PERCENT	FEMALE +ve	PERCENT
Kafr el Sheikh	819	224	27.35	48	5.86
Dosouk	106	17	16.03	10	9.46
Shebein	236	34	15.25	15	6.36

CENTRE	TOTAL	MALE +ve	PERCENT	FEMALE +ve	PERCENT
Biala	34	4	11.76	5	14.70
Ismailia	207	56	27.05	22	10.62
Port Said	421	62	14.72	15	3,56
Zagazig	356	86	24.15	25	7.27
Shoubrahoor	162	45	27.77	9	5.61
Menouf	59	8	13.55	5	8.47
Ashmoun	122	6	4.91	8	6.55
Fakous	74	11	14.74	5	6.47
Minia el Kamh	67	13	19.40	5	7.46
Manzala	42	4	9.52	2	4.76

The sex distribution of patients yielding atypical mycobacteria strains in these centres is shown as follows:

CENTRE	TOTAL	MALE	FEMALE
Zagazig	1	1	—
Ismailia	4	2	2
Port Said	1	1	—
Total	6	4	2

The incidence of these atypical mycobacteria strains among positive cultures is presented as follows:

CENTRE	+ve	ATYPICAL	PERCENT
Zagazig	111	1	0.9
Ismailia	78	4	5.1
Port Said	77	1	1.3

No atypical mycobacteria strains were isolated from the other centres of this group.

Approximately about 70 per cent of patients submitting specimens of sputum to the central laboratory were males.

The atypical mycobacteria strains isolated were differentiated from virulent tubercle bacilli initially by their colour. This varies from yellow to orange, deep orange or even red which is not met with in tubercle bacilli.

Strains of group I photochromogens possess the most characteristic property of developping a bright yellow pigment within 24 hours after exposure to light.

Strains of group II photochromogens are usually yellow or orange when grown in absence of light. It is observed that if the strains of this group are cultured from the beginning in the presence of continuous light, they give a reddish colour.

Two kinds of solid medium are used, namely Lowensteins Jensen medium and Ogawa medium.

Two cultures of each strain are grown on these media. One culture is wrapped in black paper while the other is left in continuous light. This was done by using internally illuminated incubators.

The wrapped cultures are left until the unwrapped shows good growth.

Another distinctive characteristic of these atypical mycobacterial strains is their cellular and colonial morphology in cultures grown on solid media for three to four weeks, as presented in the following table.

Using fluid media; the atypical strains were cultured on Dubos medium containing citrated human plasma. Smears were prepared and stained by the Zeihl Neelsen technique. They were examined for any tendency on the part of the bacilli to be aligned in parallel rudimentary cord formation.

Most strains lacked the ability to form serpentine cords.

Also the modified N. medium helped to differentiate the atypical strains from the virulent mycobacteria. A tube of N. medium inoculated from a suspension of the atypical mycobacteria was incubated for four days only. If growth occurs, a second tube of N. medium was inoculated from the first tube. Acid fast bacilli of the atypical mycobacteria can be seen in smears prepared four days after the second tube inoculation.

Morphology:

The isolated atypical strains are characterised by their irregular morphology. Coccoid, very short, long, beaded bacilli were met with.

The strains of group I photochromogens have large cells. They are usually beaded and tend to be arranged in cords. Whereas strains of group II photochromogens have cells varying in size and show no tendency to cord formation.

The effect of temperature on growth was a very important characteristic. Almost all the atypical mycobacteria strains showed a big ability to grow at room temperature as well as at 25°C.

All strains of group I showed good growth at 37°C after about two weeks or a little more. They exhibited slow growth at about 25°C, and no growth at 45°C. M. marinum can grow at 24°C and 32°C, but fails to grow at 37°C. M. ulcerans can grow at 32°C but fails to grow at 25°C and 37°C.

The temperature growth rate relationship is very useful in identifying M. marinum and M. ulcerans.

Rate of growth:

The most characteristic difference of the atypical strains is their short growth ~~rate~~ time. They can grow faster than the virulent tubercle bacilli. Suspensions of the atypical strains were prepared and cultured on fresh Lowenstein's and Ogawa media. They are incubated at 25°C , 37°C and at 45°C in internally illuminated incubators.

The evidence of growth is ~~remarked~~ recorded during the continuous inspection of the cultures. A big number of these strains showed growth at 25°C within three weeks or even less. It should be noted that some strains exhibited their characteristic growth rate only after subculture.

The following tables show the important morbid characteristics of the collected atypical mycobacteria.

Twenty four strains belong to group II and four strains belonging to group III were isolated during this period.

These latter strains are characterized by their rather slow growth. They may be rough or partially so . After repeated subculture, transformation to more eugonic type may occur.

These strains may resemble avian tubercle bacilli, however they are differentiated by the fact that the avian tubercle bacilli can grow at 45°C and their pathogenicity to the fowl and rabbits. On the other hand all atypical strains of group III reduce tellurite in three days and are Tween hydrolysis negative.

Cairo Centres	STRAINS	Male	Female	Chromogen			B. Length			Emulsific.		Col.Morph.	
				Y.O.	O.	D.O.	Sh.	M.L.	L	+	-	S.	R
A.Maher	2	2	-	I	I			2			2		2
Boulak	1	1	-		1		1				1		1
Khalifa	2	1	1	1	1			2		1	1	1	1
B.Sharia	3	3	-		1	2	1	2		1	2	1	2
Marg	2	1	1	2			1	1		1	1	1	1
Mataria	1	1	-		1			1		1		1	
S. Zeinab	1	-	1	1			1				1		1
Nafssia	2	2	-		1	1		1	1	2		2	
Heliopolis	1	1	-	1			1			1		1	

Guiza Centres	Strains	Male	Female	Chromogen			B.length			Emulsific.		Colon.Morph.	
				Y.O	O	D.O	SH.	M.L.	L	+	-	S.	R.
Guiza D.	2	2	-	1	1	-	1	1			2	1	1
Imbaba	5	3	2	3	2		1	2	2	3	2	3	2
Total	7	5	2	4	3		2	3	2	3	4	4	3

Y.O = yellow orange

O = orange

D.O = deep orange

Sh. = short bacilli

M.L. = middle length

L. = long bacilli

+ = easily emulsified

- = not easily emulsified

S = smooth colonies

R = rough colonies

Other Centres	Strains	Male	Female	Chromogen			B. Length			Emulsif.		Colon. Morph	
				Y.O	O	D.O	Sh	M.L.	L.	+	-	S.	R
Zagazig	1	1	-			1	1				1		1
Ismailia	4	2	2	1	3		1	3		3	1	4	
Port Said	1	1		1				1			1	1	
Total	6	4	2	2	3	1	2	4		3	3	5	1

Y.O. = yellow orange

O. = orange

D.O. = deep orange

Sh. = short bacilli

M.L. = middle length

L. = long bacilli

+ = easily emulsified

- = not easily emulsified

S = smooth colonies

R = rough colonies

Animal inoculation

Practically the guinea pig has no resistance to infection with virulent tubercle bacilli. These bacilli cause progressive fatal disease, while the atypical mycobacteria are not pathogenic to this animal species. Moreover the guinea pig tends to tolerate the commensals of the specimen which easily contaminate cultures. So less severe decontamination techniques are employed for guinea pig inoculation. This favours a higher survival rate of the mycobacteria.

Young males weighing about 250-350 gm. are used. Cultures of the atypical mycobacteria strains grown on solid media are emulsified in saline and prepared for inoculation. The animal is inoculated subcutaneously into the lower right flank. To guard against loss of test by premature death of animal, two guinea pigs are usually inoculated for each strain.

Animals are killed six weeks after inoculation. The findings vary from completely negative to the development of some changes. Some strains produce local abcesses which may last several weeks. Others may develop small abcesses in the regional lymph nodes.

Most strains produce self healing granulomatous lesions and are slightly pathogenic to the guinea pig.

Primary cultures are used for inoculation.

It was common to find some minute lesions and necrosis in visceral lesions. In smear preparations no acid fast bacilli were seen. A degree of pneumonia was sometimes seen in the lungs.

Drug susceptibility:

Patterns of resistance are of considerable value in diagnosis.

In our study, the drug susceptibility tests of the isolated atypical mycobacteria strains was done on primary cultures.

Ogawa and Lowenstein's Jensen media were the chosen solid media used in performing these tests.

It is essential to use a culture of well known drug susceptibility as a control in every test to demonstrate the activity of the drugs in the prepared media. M. tuberculosis strain H 37 RV is satisfactory for this purpose.

Most colonies of the isolated mycobacteria strains were easily emulsified with the exception of a few strains which were not easily dispersed. It is important to avoid clumps and to have the suspension perfectly homogenised.

The result of reading these sensitivity tests is determined by comparing the amount of growth on control and drug containing media. It is essential that the inoculum for each culture be of a demonstrated uniformity.

The concentration of the drug inoculum to be used is supposed to give at least from 50 to 100 colonies on control tube.

Heavy concentrations should be avoided so that the inoculum does not exceed a certain concentration, otherwise a confluent growth covers the surface of control tubes and consequently a very small proportion of drug resistant organisms may result in the presence of a large number of colonies on the drug containing media insinuating more resistance than that which is actually present.

Solutions of the antituberculous drugs are prepared and incorporated in the media.

Streptomycin, para amino salicylic acid and isonicotinic hydrazide, antituberculous drugs of the first line, are used together with other drugs of the second line namely, thiosemicarbazone, cycloserine, parasinamide and rifampicin.

Three concentrations of each drug are employed. They are presented in the following:

Drug	Ist conc.	2nd conc.	3rd conc.
Streptomycin	6	20	200 mg/ml
P A S	1	5	25 mg/ml
I N H	0.2	1	5 mg/ml
Thiosemicarbazone	2	10	50 mg/ml
Cycloserine	10	20	40 mg/ml
Pyrazinamide	10	20	40 mg/ml
Rifampicin	4	8	32 mg/ml

As the drug containing media should not be stored more than four weeks, we prepare only the quantity required for this limited period.

Media containing the different drug concentrations together with two tubes containing media free from the drug are inoculated with 0.1 ml of the standardised bacterial suspension. The drug free media tubes are the controls. The inoculum is spread as evenly as possible over the whole surface of the tubes.

The results of the susceptibility are reported by comparing the amount of growth on the drug containing media and the drug free media. A highly sensitive strain exhibits growth only on the control tubes whereas a resistant strain shows growth in the tube containing the highest concentration of the drug.

As the result of the drug susceptibility is based on comparing the amount of growth on control and drug containing media, the inoculum should be of a precise uniformity for each culture. Homogenisation of the inoculum and avoiding large clumps is important.

The inoculum must be of a concentration that can give rise to 50-100 colonies on the control media, but it should not be too heavy to result in a confluent growth, for in this case a very small proportion of drug resistant strains may result in the presence of a large number of colonies on the drug media and may suggest more resistance than that which is actually present.

In this work the susceptibility of the isolated strains was described as slightly, moderately or highly resistant depending on the presence of growth. Growth on drug media not exceeding ten colonies is ignored if the control media tubes reveal a confluent growth.

If there is growth on all the drug containing media including the highest concentration, the result is recorded as highly resistant.

When the growth appears on the first and second drug concentrations and does not appear on the third, namely, the highest concentration, the result is recorded as slightly sensitive

If the growth shows on only the first tube, and does not appear on the second and third concentrations, the result is recorded as moderately sensitive.

The strain is recorded as sensitive if no growth appears even on the lowest drug concentration.

The results of the sensitivity tests of the isolated atypical mycobacteria are presented as follows:

	STREP	PAS	INH	THIO	CYCL	PZA	RIFAMP
Sensitive	2	0	0	2	4	0	4
Slight.resist.	2	2	2	5	4	0	3
Moderat.resist.	4	1	3	5	5	1	6
Highly resist.	20	25	23	16	15	27	15
Total	28	28	28	28	28	28	28

Almost all the isolated atypical strains are resistant to para amino salicylic acid and pyrazinamide.

Strains of group II are less susceptible to the antituberculous drugs of the first line: streptomycin, paraaminosalicylic acid and isoniazide.

Strains of group III are also resistant to the same drugs although some are susceptible.

Both groups show a varied susceptibility to cycloserine and rifampicin more than to other antituberculous drugs.

Again the non pigmented strains are more resistant than the pigmented strains.

Cytochemical tests:

Some useful cytochemical tests were used to differentiate the isolated atypical mycobacteria. It is evident that most of these strains may be identified by their rate of growth, pigmentation and colonial morphology, but the use of these biochemical tests is a further step upon which we can rely in our identification. Moreover, they are considered a rapid means of diagnosis compared with the animal inoculation and culture techniques.

Cytochemical neutral red test:

The atypical mycobacteria are neutral red negative. Only virulent strains of mycobacterium tuberculosis take up the neutral red in alkaline buffer solutions, whereas avirulent strains fail to do so. It is also noticed that neutral red +ve cultures of mycobacterium tuberculosis produce tuberculosis by guinea pig inoculation, whereas the atypical mycobacteria which are neutral red negative, produce no lesions by animal inoculation.

The depth of colour obtained varies according to the size of the inoculum, the time of incubation and the bacterial contaminants.

Arylsulfatase test:

This test is performed mainly for strains of group IV, rapid growers atypical mycobacteria. These organisms are able to split phenolphthalein from tripotassium phenolphthalein sulphate. A positive test is indicated by a pink colouration while a negative test presents no change in colour.

Growth on Mac Conkey agar:

Some rapid growers, M. fortuitum, can grow on Mac Conkey agar and this helps to differentiate them from other rapidly growing mycobacteria.

Iron uptake:

In this test only the rapid growers, such as M. fortuitum and M. phlei are positive. This is recorded by the rusty brown colour which appears on the colonies and the tan discolouration of the medium.

Catalase test:

When several colonies of the mycobacteria are suspended in saline phosphatase buffer at pH 7 and placed in a water bath at 68°C for 20 minutes, a positive result definitely indicates a species other than tubercle bacilli. A high or even a moderate activity is diagnostic for atypical mycobacteria strains.

Niacin test:

This test is very useful to differentiate the atypical strains from the tubercle bacilli. M. tuberculosis is characterised by its abundant niacin production, more so than any other mycobacteria. Niacin negative M. tubercle bacilli are exceedingly rare, while on the other hand +ve niacin with strains other than M. tuberculosis is also rare. In case of a negative result associated with evidence of M. tuberculosis, repeat the niacin test using a more luxuriant growth.

There is no advantage in doing niacin test on strains which are scotochromogenic, photochromogenic, or rapid growers. However the possibility of encountering individual niacin +ve strains of group III non-pigmented, must not be ignored.

Nitrate reduction test:

The nitrate reduction test is of considerable significance in the identification of slow growing mycobacteria. M. tuberculosis and M. Kansasii are strongly nitrate reductase positive, whereas the atypical strains of group II scotochromogens and strains of group III, Battey, are negative or at most very weakly positive.

Tellurite reduction test:

This test is useful to identify rapid growers, as the colourless potassium tellurite salt is reduced to black metallic tellurium within three to four days. This distinctive property served to identify some strains of group III

Tween 80 hydrolysis test:

The enzymatic hydrolysis of Tween 80 releases oleic acid changing of neutral red. The result is recorded as positive when the colour changes from the original amber to a pink or red colour.

Most strains of M. Kansasii are positive within 5 days. Some scotochromogens and Battey strains are negative whereas the M. tuberculosis cultures are positive in 10 - 20 days.

In the period from April 1972 to August 1976 approximately 61700 samples of sputum were cultured in the central tuberculosis laboratory in Cairo. 22.68% were positive for tubercle bacilli. 346 strains of atypical mycobacteria were isolated giving an incidence of 2.47 per cent among the positive cultures.

314 chromogenic mycobacteria strains comprising both group I and group II were identified in this collection. 36 of these strains belonged to group I, while 278 fall under group II

23 strains of atypical mycobacteria isolated were found to belong to group III and 9 strains belonged to group IV.